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CONFORMATIONAL EFFECTS IN THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

FURTHER STUDIES OF THE REVERSED-PHASE CHROMATOGRAPHIC BEHAVIOR OF RIBONUCLEASE A

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SUMMARY

This paper is a continuation of an examination of the conformational effects of ribonuclease A (RNase A) in reversed-phase liquid chromatography. RNase A is a particularly good example of reversible conformational refolding during chromatographic elution. Absorbance ratio measurements with a photodiode-array detector are consistent with a two-state model in which an early eluted broad band is associated with the folded or native state and a late eluted sharp band is associated with an unfolded state. By varying the mobile phase flow-rate while maintaining the mean capacity factor (k') of the protein in gradient elution constant, the apparent rate constant of refolding in the mobile phase was measured and found to be comparable with that reported in the literature. It has been further shown that, as the gradient steepness parameter b or mean k' value is altered, the apparent rate constant also changes, as a result of variation in mobile phase composition. The apparent rate constant as a function of temperature was also found to reach a maximum at 20°C. Based on the results of this model system, it is possible to predict optimum conditions for elution of species with sharp elution peaks when reversible refolding takes place in the column.

INTRODUCTION

Conformational changes for proteins in either the stationary or mobile phase are known to play an important role in the high-performance liquid chromatographic (HPLC) separation of proteins. Such changes, if reversible during chromatographic elution, can lead to severely distorted and broadened peaks. Alternatively, if the kinetic processes of conformational change are slow or irreversible during elution, multiple peaks can be observed. Such behavior can significantly alter chromatograph-

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ic performance, leading to complex, poorly separated and poorly reproducible peaks. Recent papers have dealt with this behavior in detail[1–7].

When conformational changes occur in an HPLC column, this phenomenon must be explored for several reasons. First, in certain chromatographic procedures, particularly in reversed-phase liquid chromatography (RPLC), protein denaturation or unfolding is often observed[8]. Through a study of protein behavior, rational choices of column conditions can be made to achieve high performance and, in certain cases, to yield biologically active species. Secondly, conformational behavior can, in principle, be used to probe chromatographic distribution or adsorption[2]. Thus, examination of such protein behavior in a chromatographic system is clearly of importance in HPLC.

Recently, we reported on the reversible conformational changes of ribonuclease A (RNase A) in RPLC[1]. RNase A represents a good example for the study of such effects, since the protein is well defined. In addition, RNase A has been extensively used as a model protein for studies of refolding and unfolding in solution[9–15].

In our previous work[1], by using an *n*-butyl-bonded stationary phase and a gradient mobile phase from 10 m*M* orthophosphoric acid (pH 2.2) to 1-propanol–10 m*M* orthophosphoric acid (45:55, v/v) at room temperature, we showed that a broad band, followed by an overlapping late-eluted sharp peak was obtained. This unusual band shape collapsed into a single, sharp peak at 37°C. Based on measurements of absorbance ratio A_{288}/A_{254}, it was suggested that the broad band at 25°C represents the folded or native state of RNase A and the late-eluted, sharp peak an unfolded state. In addition, the band shape was observed to be altered as the mobile phase flow-rate was changed at 25°C (while the gradient steepness parameter, b,[16] was kept constant). At a high flow-rate, the broad band was small, increasing in magnitude relative to the late-eluted peak as the flow-rate was reduced. These results suggested that the unfolded RNase A was renatured in solution with a half-life comparable to the time that the protein spent in the mobile phase during its travel through the column (*i.e.* the inert peak time). It is well known that the unfolding of RNase A is reversible[9,17], and the results appear reasonable, particularly in light of the fact that off-line circular dichroism studies at 25°C revealed that the protein was in the native state under the elution condition[1].

This paper is a continuation of the above-mentioned studies on RPLC of RNase A. Measurements of the first-order rate constant of refolding as a function of the gradient steepness parameter, b, at a given column temperature and as a function of temperature are presented. The results are shown to be consistent with the previous model of refolding in the mobile phase[1]. This work forms the basis of an understanding of the chromatographic behavior of RNase A and, more generally, of proteins undergoing reversible conformational changes under gradient elution conditions.

EXPERIMENTAL

The measurement of refolding rate constants was carried out with a DuPont 8800 liquid chromatograph (DuPont Instruments, Wilmington, DE, U.S.A.) and a rapid-scanning photodiode-array detector (HP 1040A, Hewlett-Packard, Avondale, PA, U.S.A.). Chromatographic and spectral data were acquired and evaluated with

an HP 85B computer (Hewlett-Packard). In order to minimize the temperature difference between the mobile phase and column, a long, coiled tube with a volume of roughly 6 ml was inserted between the pump outlet and injector. The tubing and column were immersed in a water bath.

A *n*-butyl-bonded phase was prepared in our laboratory from 7-μm Vydac (Separations Group, Hesperia, CA, U.S.A.) spherical silica with 300 Å pore diameter and 78 m^2/g surface area. The bonded phase was endcapped with hexamethyldisilazane. The carbon content was determined by elemental analysis to be 2.56%. HPLC-grade water and 1-propanol were products of Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Bovine pancreatic ribonuclease A was purchased from Sigma (St. Louis, MO, U.S.A.) and was used as received. Mobile phase A was 10 m*M* orthophosphoric acid (pH 2.2) and mobile phase B consisted of 1-propanol–10 m*M* orthophosphoric acid (pH 2.2) (45:55, v/v). Both solvents were degassed by helium sparging.

RESULTS AND DISCUSSION

Fig. 1 presents the chromatographic gradient elution behavior of RNase A as a function of mobile phase flow-rate. In this experiment, the gradient rate (percentage of 1-propanol per min) was changed in the same proportion as the flow-rate in order to maintain $\bar{k}'$ [*i.e.* the capacity factor (k') as the protein passes the center of the column] constant. By this procedure and using linear solvent strength gradients, the b value, as defined in eqn. 1, is maintained constant[16]

$$b = \frac{\Delta\phi S V_m}{F t_G} \tag{1}$$

where $\Delta\phi$ is the gradient range of 1-propanol, S is the solvent strength parameter or slope of the log k' *versus* volume fraction of 1-propanol plot, V_m is the dead-volume of the column, t_G the time of linear gradient operation, and F the mobile phase flow-rate.

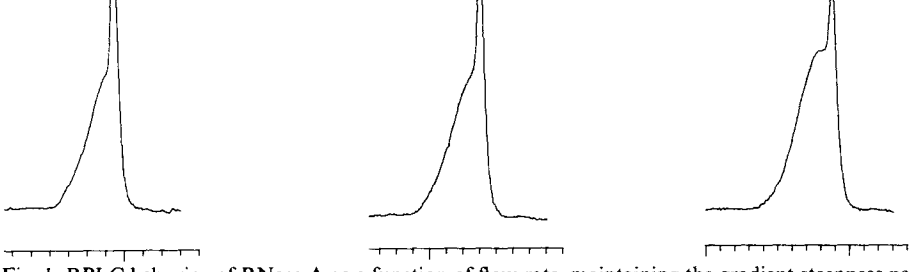


Fig. 1. RPLC behavior of RNase A as a function of flow-rate, maintaining the gradient steepness parameter b constant = 0.66. F = 1 ml/min (A); F = 0.8 ml/min (B); F = 0.6 ml/min (C). Conditions: mobile phase A, 10 m*M* orthophosphoric acid (pH 2.2). Mobile phase B, 1-propanol–10 m*M* orthophosphoric acid (pH 2.2) (55:45, v/v). Gradient: segment 1, 15 min, isocratic, mobile phase A; segment 2, 25 min, 0–85% mobile phase B; segment 3, 5 min, 85% mobile phase B. Column: 7 μm C_4 bonded phase, 100 × 4.6 mm I.D. Sample size: 125 μg RNase A.

It can be observed in Fig. 1 that the lower the flow-rate, the more pronounced is the broad band and simultaneously, the lower is the peak height of the sharp, late-eluted band. In this experiment, the time the protein spends traveling through the column varies inversely with the flow-rate. Thus, for $F = 0.5$ ml/min, the protein will spend twice as long in the mobile phase as at a flow-rate of 1 ml/min.

We have previously observed that for a given set of chromatographic conditions in the temperature range of 25 to 40°C, the peak shape is independent of the time RNase A spends on the bonded phase surface prior to elution. Thus, apparently the phenomena in Fig. 1 are occurring in the mobile phase, since none of the kinetically controlled surface processes observed for other proteins[2], are found for RNase A in this system ($T > 20$°C). This conclusion is reinforced by the fact that several different *n*-butyl-bonded phases on different silicas (LiChrospher[1] and Vydac) yield

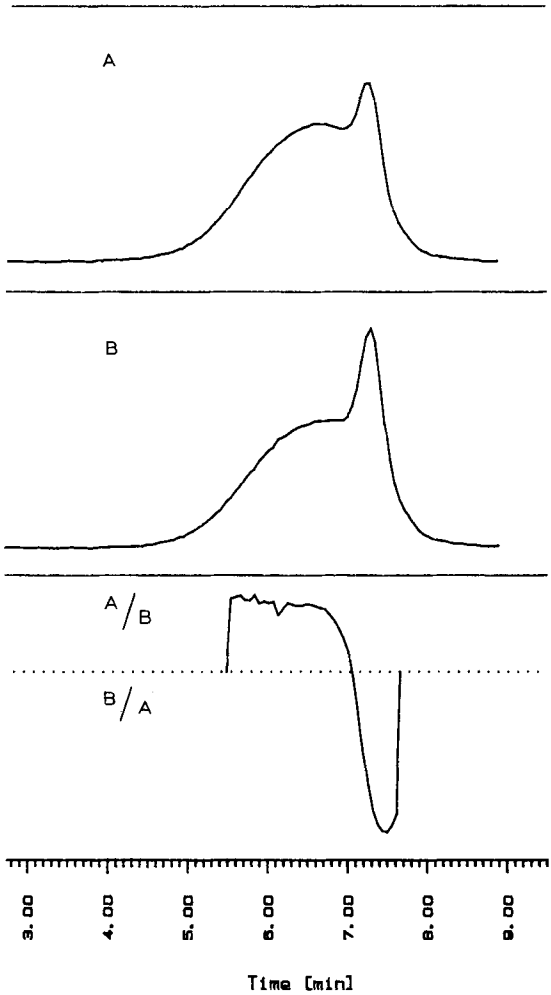


Fig. 2. RPLC behavior of RNase A as a function of detection wavelengths at 287 nm (A) and 254 nm (B). Absorbance ratios A/B or B/A from the HP 1040A photodiode-array detector. See Fig. 1 for other conditions.

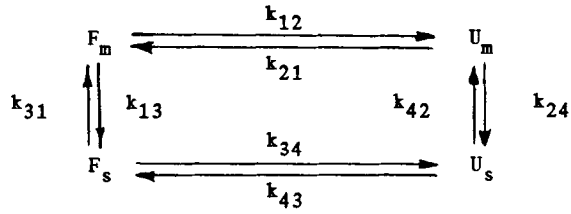
the same chromatogram for the same gradient and column temperature above 20°C. In order to ensure the absence of the native state on the bonded phase at temperatures above 20°C, we have included an isocratic hold of 15 min. before starting the gradient. It is to be noted that below 20°C a complete loss of the native state on the surface is not likely to take place (see below).

Recently, we have been examining the use of a rapid-scanning detector for the on-line detection of conformational changes during HPLC of proteins. It is important to characterize bands as they are eluted from the column, since dynamic processes can continue to occur in solution upon fraction collection. This is particularly true in the case of RNase A, where reversible kinetic processes in the mobile phase of the order of seconds are known to occur.

Fig. 2 illustrates the use of the photodiode-array detector to provide information on the conformational states of RNase A in the two bands. For the top chromatogram a wavelength of 287 nm was used for detection, whereas the middle chromatogram is based on the use of 254 nm. Previous studies with difference spectroscopy[1] have shown that these two wavelengths can be applied to the elucidation of native and thermally denatured states. The 287-nm signal represents the change in the unfolding process of tyrosine amino acid residues from the interior to the surface of the protein and the subsequent solvation of these residues by the mobile phase.

The bottom plot in Fig. 2 shows the 287 nm/254 nm absorbance ratio across the band. It can be observed that for the broad peak the absorbance ratio is roughly constant but it significantly changes for the sharp, late-eluted peak. This behavior is suggestive of a two-state model in which, based on the difference spectroscopy results[1], the first band represents the folded conformational state and the second band an unfolded conformational state. It is known that intermediates occur in the pathway of refolding of RNase A[11,13–15]. However, their lifetimes are of the order of milliseconds[13], and given the time resolution of the chromatographic process of the order of seconds, it is impossible to observe such intermediates in these experiments.

Based on Figs. 1 and 2 and on the results of our previous paper[1], we can propose the following model of conformational changes of RNase A during RPLC:



where F represents the folded state (corresponding to native) and U an unfolded state, subscripts m and s represent mobile and stationary phase, respectively, and k the appropriate rate constant. In this model, as noted above, we have omitted intermediates; however, they can obviously be included, if their lifetimes are sufficient.

Equilibria can exist between the mobile and stationary phase and within the both phases. At temperatures of 20°C and above, we assume that the native species

is driven into an unfolded state on the bonded phase, *i.e.* K_s is large, where $K_s = k_{34}/k_{43} = U_s/F_s$. This assumption would then mean that only unfolded RNase A exists on the bonded phase surface under these conditions. Below 20°C, we can no longer neglect folded state protein occurring on the bonded phase surface.

As the solvent strength of 1-propanol becomes sufficient to desorb U_s, unfolded protein slowly refolds in the mobile phase to native state material, *i.e.* K_m is finite, where $K_m = k_{21}/k_{12} = F_m/U_m$. Once F_m forms, it should pass through the column unretained, since the solvent strength for elution of this folded species has been surpassed in 1-propanol concentration.

Kinetically controlled equilibria have been examined in detail by Melander *et al.*[3]. However, their study was based on isocratic elution. Gradient elution, as required for the HPLC of proteins, significantly complicates the situation. For example, the capacity factors and the equilibrium constants become dependent on gradient conditions. In the next section, we determine from gradient elution chromatography the rate constant for refolding of RNase A in solution under a variety of conditions in order to provide insight into the factors that control the rates of reversible refolding and thus peak shape in gradient elution.

Refolding rate constants

The apparent rate constant for refolding in the mobile phase, k_f, can be determined by measuring the peak height of the late-eluted peak (corresponding to the unfolded form) as a function of mobile phase flow-rate while maintaining b constant. From eqn. 1, this experiment requires that t_G varies inversely with F. For linear solvent-strength conditions, a constant b value means that the mobile phase composition is maintained constant as the protein passes the midpoint of the column (*i.e.*, $\bar{k}'$ is constant). If t_G is not varied with F, then the peak-height change that is measured

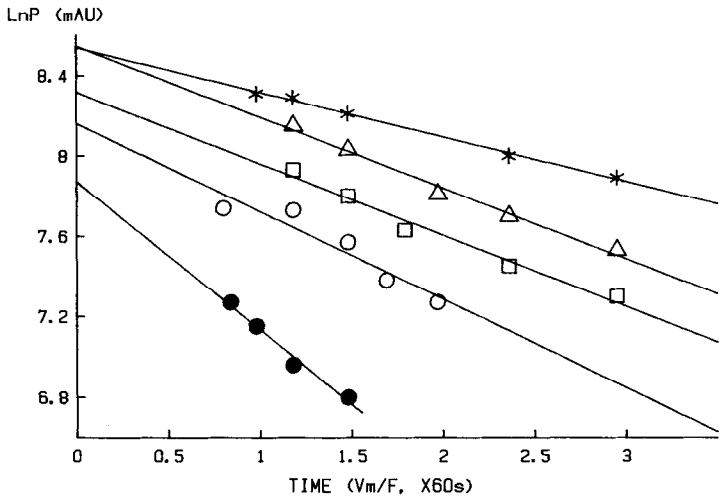


Fig. 3. Plots of ln P *vs.* inert peak time for the refolding of RNase A at various b values. P = Peak height of the late eluting (unfolded) peak; T = 20°C. Key to symbols: ●—●, b = 0.25, $k_f = 12.3 \cdot 10^{-3}$ s^{-1}; ○—○, b = 0.41, $k_f = 8.8 \cdot 10^{-3}$ s^{-1}; □—□, b = 0.55, $k_f = 6.4 \cdot 10^{-3}$ s^{-1}; △—△, b = 0.66, $k_f = 6.0 \cdot 10^{-3}$ s^{-1}; *—*, b = 0.82, $k_f = 3.8 \cdot 10^{-3}$ s^{-1}.

will be a result of the time the protein spends in the mobile phase along with the change in $\bar{k}'$.

Fig. 3 shows the linear behavior of the log peak height *vs.* the time spent by RNase A in the mobile phase at various b values at a column temperature of 20°C. Separate studies of standard proteins not undergoing reversible conformational changes revealed that the peak-height variation over the flow-rate range examined was less than 5% of the changes observed for the RNase A peak. In addition, the peak-height change for RNase A at 40°C (only unfolded protein exists at this temperature under the chromatographic conditions)[1] over the flow-rate range was again less than 5%. For this work, we have chosen to ignore this small correction.

The plots and caption of Fig. 3 indicate that the apparent rate constant of refolding k_f is increased as the b value is decreased. This result can be readily observed in Fig. 4, which shows RNase A chromatograms at a constant flow-rate of 1 ml/min

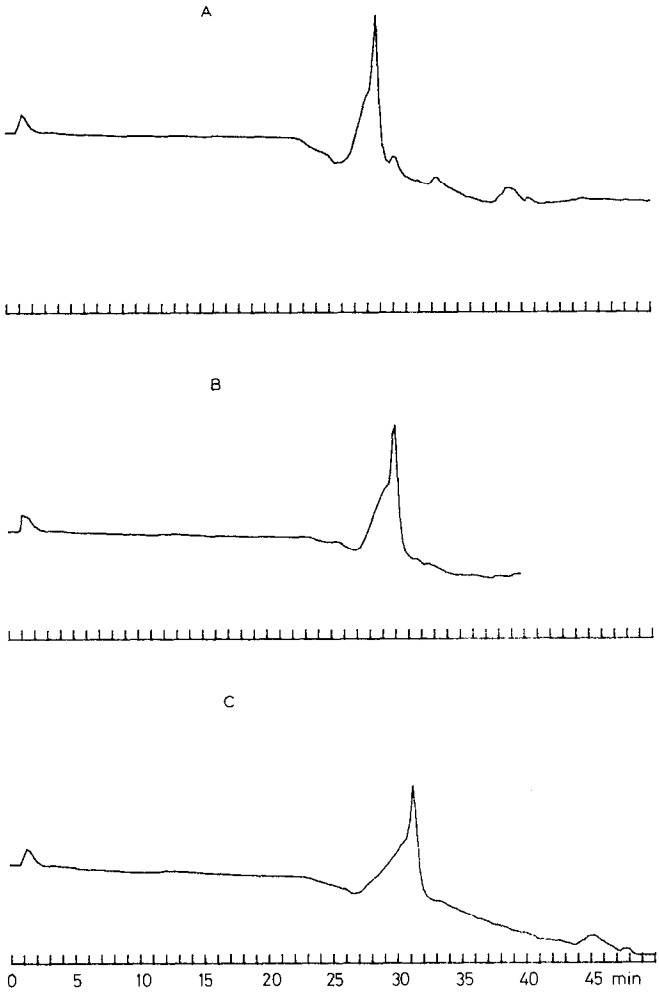


Fig. 4. RPLC chromatograms of RNase A as a function of the b value at constant flow-rate of 1 ml/min. b values: A, 0.82; B, 0.66; C, 0.55. Other conditions: see Fig. 1.

with b varying from 0.55 to 0.82. For the highest b value of 0.82, the amount of broadening of the first peak is significantly less than for a b value of 0.55. Thus, in gradient elution, the gradient conditions along with the flow-rate would appear to affect the chromatographic results. Since b is inversely proportional to $\bar{k}'$ (ref. 16), the results of Fig. 3 indicate that the apparent rate constant for refolding increases as $\bar{k}'$ increases.

We can understand the reasons for this behavior from an examination of Fig. 5 which is a plot of k_f *vs.* b and *vs.* $\bar{\phi}$, the composition of 1-propanol as the solute passes the midpoint of the column. It is readily apparent that as b increases the volume fraction of 1-propanol also increases.

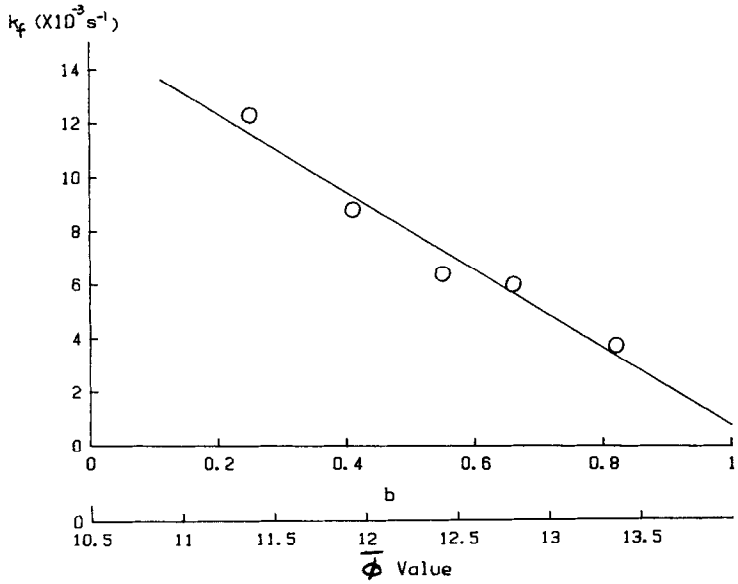


Fig. 5. Apparent refolding rate constants for RNase A from Fig. 3 *vs.* gradient steepness parameter b and *vs.* $\bar{\phi}$, the volume fraction of 1-propanol as the protein passes the midpoint of the column.

The apparent rate constant for refolding, k_f, will represent refolding in the mobile phase, if refolding in the stationary phase is negligible, *i.e.*, k_{43} is negligible. This assumption is reasonable, particularly above 20°C, since no folded-state protein appears to exist on the bonded-phase surface above this temperature. On this basis, the apparent rate constant can be related to the equilibrium constant K_m, if the activated state is native-like[17]. K_m is expected to be lower with increasing 1-propanol concentration[17]. Hence, the apparent rate constant should be decreased as K_m is decreased. From this discussion, it is possible to understand why an increase in the b value decreases the apparent rate constant of refolding.

Let us consider next the value of the apparent rate constant. As seen in Fig. 3, for $b = 0.25$, k_f is equivalent to $1.2 \cdot 10^{-2}$ s^{-1} or a half-life of 56 s. For $b = 0.81$, the rate constant becomes $3.8 \cdot 10^{-3}$ s^{-1} or a half-life of 180 s. These half-lives are within the range of the inert peak time which, at a flow-rate of 1 ml/min, is 71 s. As shown by Melander *et al.*[3], the Damkohler number, which is equivalent to t_0 times

k_f, will equal 0.85 for $b = 0.25$ and 0.27 for $b = 0.81$. When the Damkohler number is close to 1, peak distortion takes place[3]. Stated differently, when the relaxation time is comparable to the inert peak time, kinetic processes have their greatest effect on peak shape. Finally, the rate constants measured under different mobile phase conditions compare favorably with those published by Lin *et al.*[13]. This agreement lends further credence to the model.

We next explored the influence of column temperature on k_f. Again, because of gradient elution, care had to be taken to assess the rate constant effects correctly. As before, we determined k_f by varying the mobile phase flow-rate and measuring of the height of the unfolded peak between 10 to 30°C. In this experiment, the b value was varied in an appropriate manner in order to keep the mobile phase composition constant as the protein reached the midpoint of the column. Thus, the b value was altered in such a way that for a given flow-rate the retention time in the gradient system remained constant. Calculations showed that this approach yielded a constant mobile phase composition as the protein passed the midpoint of the column. The variations were from $b = 0.74$ at 10°C to $b = 0.54$ at 30°C, yielding an average mobile phase composition of roughly 13% (v/v) 1-propanol.

Fig. 6 displays the rate constants as a function of temperature. It is readily apparent that the rate constant reaches a maximum at 20°C and then drops sharply to 30°C. The rate behavior shown in Fig. 6 is expected for a system close to equilibrium and has been observed in refolding processes in solution[17]. K_m is known to have a maximum as a function of temperature for refolding[17,18]. Assuming, as above, a relationship between the activation energy for this process and K_m, the results in Fig. 6 are expected.

Below 20°C, a peak shape emerged which was different from that observed at

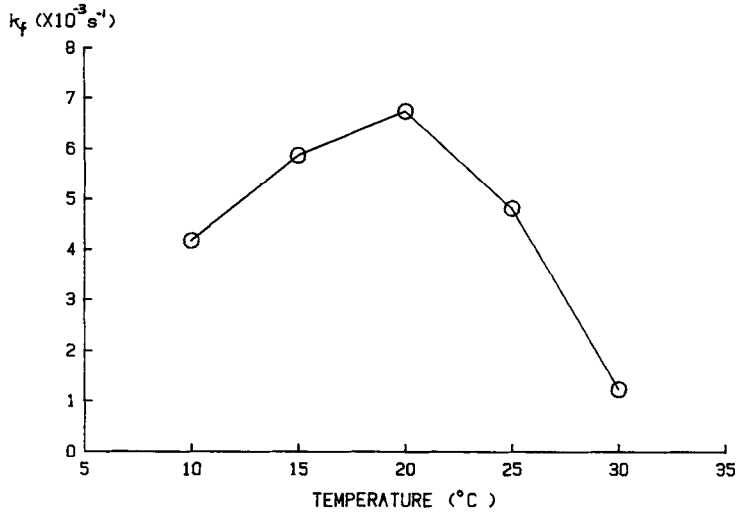


Fig. 6. Apparent refolding rate constants for RNase A as a function of column temperature. See text for conditions.

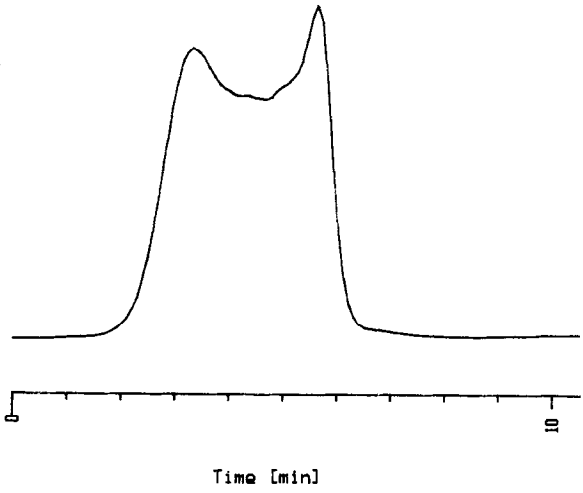


Fig. 7. RPLC chromatogram of RNase A at 15°C, F = 1 ml/min, to illustrate appearance of a third peak. Other conditions: see Fig. 1.

20°C and above. Fig. 7 illustrates chromatography at 15°C in which a third, overlapping peak is seen, eluted ahead of the other two bands. This first peak increased in relative size as the column temperature was lowered. In addition, on-column incubation resulted in a decrease in this peak. The first peak presumably represents native RNase A not denatured on the bonded phase. Thus, low temperature conditions appear to yield a mixture of folded and unfolded material on the bonded phase surface. In addition, the unfolded material, upon release from the stationary phase, appears to follow the mobile phase behavior observed above 20°C.

CONCLUSIONS

RNase A represents a good model protein for studying reversible kinetic processes in gradient elution. Since the relaxation time for refolding is comparable to the time spent by the protein in the mobile phase, as it travels through the column, peak distortion is observed. By varying the mobile phase flow-rate while b (and thus the average mobile phase composition) is kept constant, it has been possible to determine the apparent first-order rate constants for refolding via measurement of the change of peak height of the unfolded form.

Gradient elution complicates the study and understanding of rate processes in LC, because changes in gradient conditions can alter the mobile phase composition and, thus, the apparent rate of refolding. Our measurements have shown that, as b increases at constant column temperature, the apparent refolding rate constant decreases, and the band shape is appropriately affected. This trend is a consequence of the fact that at higher b values, the average 1-propanol concentration is greater, leading to less stabilization of the folded state. The equilibrium constant for mobile phase refolding, K_m, will decrease with higher b values. Assuming that the equilibrium between the activated state and the unfolded state changes in a similar

fashion to K_m[17], then the measured rate constant for refolding should also decrease.

The role of column temperature is understandable on this basis as well. Thus, a maximum in K_m as a function of temperature is well documented for a refolding process[17,18]. The maximum in k_g with temperature (see Fig. 6) may arise from this K_m variation with temperature.

It is clear that control of peak shape in protein HPLC, when conformational changes occur, requires an understanding of the equilibria and kinetic processes involved. Methodology, such as the on-line spectroscopy used in this work (Fig. 2), needs to be developed further to aid in the recognition of conformational changes. Once the causes of distorted peak shape or of multiple peaks are understood, rational approaches for optimized chromatographic separation can be developed.

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